Effect of cellulose as co-substrate on old landfill leachate treatment using white-rot fungi

EFFECT OF CELLULOSE AS CO-SUBSTRATE ON OLD LANDFILL LEACHATE TREATMENT USING WHITE-ROT FUNGI

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Abstract

Conventional wastewater treatment technologies are ineffective for remediation of old LandFill Leachate (LFL), and innovative approaches to achieve satisfactory removal of this recalcitrant fraction are needed. This study focused on old LFL treatment with a selected fungal strain, *Bjerkandera adusta* MUT 2295, through batch and continuous tests, using packed-bed bioreactors under non-sterile conditions. To optimize the process performance, diverse types of co-substrates were used, including milled cellulose from beverage cups waste material. Extracellular enzyme production was assayed, in batch tests, as a function of a) cellulose concentration, b) leachate initial Chemical Oxygen Demand (COD) and Soluble COD (sCOD), and c) co-substrate type. Bioreactors were dosed with an initial start-up of glucose (Rg) or cellulose (Rc). An additional glucose dosage was provided in both reactors, leading to significant performance increases. The highest COD and sCOD removals were i) 63% and 53% in Rg and ii) 54 % and 51% in Rc.

Keywords: Bioreactors; Landfill leachate; Recalcitrant compound removal; Wastewater treatment; White-rot fungi.

1.0 Introduction

Landfill Leachate (LFL) is defined as the liquid produced by rainwater percolation in landfill waste layers. Since sanitary landfilling is one of the most common methods for disposing Municipal Solid Waste (MSW) (Nghiem *et al*., 2016), the generation of LFL cannot be prevented (Ghosh *et al.*, 2014a). LFL contains recalcitrant organic compounds that standard biological processes are unable to efficiently degrade (Verdrenne *et al.*, 2012; Zhao *et al*, 2013a; Tigini *et al.*, 2014). Therefore, the search for innovative and sustainable technologies to reduce the impact of untreated leachate is a serious environmental concern (Jones *et al*, 2006; Ghosh *et al*., 2014b). Although the composition of LFL varies widely, depending on diverse factors including age and degree of stabilization of waste, several common features can be observed (Umar *et al.*, 2010; Razarinah *et al*., 2015), such as the presence of high ammonia concentrations, high organic loads, and the presence of inorganic compounds, including heavy metals and salts (Kamaruddin *et al*., 2014).

With landfill aging, the ratio between Biological and Chemical Oxygen Demand (BOD_5/COD) decreases due to the hydrolysis of the biodegradable organic fraction of LFL, while the non-biodegradable fraction of COD remains unchanged. In particular, three stages of LFL have been classified according to landfill age (Peyravi *et al*., 2016). Young leachate (< 5 years) presents higher concentrations of biodegradable organic loading, with a BOD_5/COD ratio > 0.3 composed mainly (about 70%) of Volatile Fatty Acids (VFA). Intermediate leachate, from 5 to 10 years, presents a BOD_5/COD ratio between 0.3, and 0.1 and its composition includes 5 to 30% VFAs, as well as humic and fulvic acids (Renou *et al*., 2008). In contrast to young LFL, old leachate (> 10 years) presents a low BOD_5/COD ratio (< 0.1) and high concentrations of refractory humic and fulvic acids as a consequence of microbial activity (Batarseh *et al*., 2010; Kalčíková *et al*., 2014; Ghosh and Thakur, 2016). However, the cut off between intermediate and old leachate is not strictly defined (Peyravi *et al*., 2016),

and often the same treatments are applied to both intermediate and old LFL (Bohdziewicz and Kwarciak, 2008).

Although biological treatments can effectively remove non-stabilized organic matter and toxic compounds in young LFL, the efficiency decreases with the age of the leachate and, generally, further physical-chemical treatment are required before discharging old LFL in receiving waters. Therefore, the achievement of sustainable technologies for old LFL treatment is still a challenge (Peyravi *et al*, 2016).

The movement of LFL into the surrounding soil, ground water, or surface water, may lead to severe pollution (Razarinah *et al*., 2015; Kumari *et al*., 2016) and thus regulations concerning LFL discharge into receiving waters are becoming more and more stringent (Renou *et al*., 2008; Peyravi *et al*, 2016). Indeed, the traditional hauling of LFL to wastewater treatment facilities can interfere with UV disinfection (Zhao *et al*., 2013b) and LFL composition can inhibit the biological treatment resulting in increased concentrations of effluents (Neczaj and Kacprzak, 2007).

For all these reasons, LFL has been regarded with particular interest as a highly polluted wastewater whose treatment is generally complex and expensive (Kamaruddin *et al*., 2014). The increasing attention on LFL treatment is clearly visible in the growing number of articles related to this topic. Over 110 articles concerning LFL treatment were published in the scientific literature between 1970 and the end of 20th century, and more than 600 have been published since the beginning of the 21st century (source ISI Web of Science).

Innovative biological treatments, such as the use of white-rot fungi, have been widely investigated, resulting effective remediation of several problematic wastewaters (Lopez *et al*., 2002), including pharmaceutical wastewater (Marco-Urrea *et al*., 2009), olive mill wastewater (Kissi *et al*., 2001), bleaching wastewater from pulp paper industries (Fang and Huang, 2002), textile wastewater (Rodriguez-Couto, 2013), and petrochemical wastewater (Palli *et al.*, 2016). Effective remediation of

soils contaminated with polycyclic aromatic hydrocarbons has also been achieved using white-rot fungi (Di Gregorio *et al*., 2014).

The use of white-rot fungi has been recently applied in combination with more common approaches, including other biological treatment methods (Gullotto *et al*., 2014), as well as physical and chemical methods (Castellana and Loffredo, 2014; Loffredo *et al.*, 2016). Recalcitrants of LFL have been in part identified as natural macromolecules including lignins, tannins, humic materials, folic acids, carbohydrates (Gourdoun *et al*., 1989) and, partially, as organic pollutants such as preservatives used in personal care products (PCPs), such as methylparaben (MP), ethylparaben (EP), propylparaben (PP), and butylparaben (BP), hormones, pharmaceuticals, halogenated hydrocarbons, and pesticides (Peyravi *et al.*, 2016). When considering the refractory fraction of LFL, the use of white-rot fungi, with their ligninolytic systems, could play an important role in its treatment (Ellouze *et al*., 2008). Effective fungal treatments are often associated with the production of extracellular ligninolytic enzymes, such as manganese-dependent peroxidases (MnP), lignin-peroxidases (LiP), and laccases (LaC) (Wesenberg *et al.*, 2003; Ellouze *et al*., 2008), all of which are expressed by white-rot fungi (Razarinah *et al.,* 2015).

Studies of fungal treatment of LFL in the scientific literature have focused, mainly, on remediation of young LFL. For example, a COD reduction of up to 90% with 50% diluted leachate has been associated with laccase activity up to 4000 U/L (Ellouze *et al.*, 2008, 2009). In contrast, inhibition of fungal enzymatic activity has been reported using 90% old LFL (Kalčíková *et al.,* 2014), although normal enzymatic activity was restored when the concentration of old LFL was reduced. Tigini *et al*. (2013) reported the association of decolourisation with ligninolytic enzymaticactivity, through batch experiments on LFL using autochthonous and allochthonous fungal strains. The authors also quantified the fungal load and ecotoxicological features of LFL (Tigini *et al*., 2014).

Although promising, the majority of the results achieved with fungal treatment on LFL have been attained in batch experiments. Only a limited number of experiments have been carried out in

119 continuous bioreactors (Ghosh *et al.*, 2014a; Saetang and Babel, 2009), and no full-scale applications
120 have been reported.

121 In this paper the treatment efficiency of a selected white-rot fungus, *Bjerkandera adusta* MUT 2295,
122 on old LFL (from a landfill site in Winnipeg, Canada) has been investigated, under non-sterile
123 conditions, through batch and continuous experiments. In particular, batch tests were performed to
124 evaluate the enzymatic activity of the fungus using glucose, malt extract or milled cellulose as co-
125 substrate under different experimental conditions including a) different cellulose concentrations and b)
126 leachate dilutions. Continuous experiments were carried out using bench-scale packed-bed trickling
127 bioreactors in which *Bjerkandera adusta* was inoculated as immobilized on polyurethane foam carriers.

128 **2.0 Material and Methods**

129 2.1 Chemicals, fungal strain, and substrates

130 All chemicals used in this study were of analytical grade and purchased from VWR Canada. The
131 fungal strain used in this study, *Bjerkandera adusta* MUT 2295, was obtained from *Mycotheca*
132 *Universitatis Taurinensis* (MUT). The strain, previously used to treat textile, tannery and
133 pharmaceutical wastewaters (Anastasi *et al.*, 2010; Spina *et al.*, 2012), was selected during previous
134 experiments (Bardi *et al*., 2016) on account of its capability of decolourizing a sample of leachate
135 (Italy) up to 40%. The color removal was associated with MnP production up to 40 U/L.

136 The strain was preserved on Malt Agar plates (MEA, glucose 20 g/L, malt extract 20 g/L, yeast
137 extract 20 g/L and peptone 2 g/L) at + 4°C and periodically inoculated in new Petri dishes to preserve
138 the colony. The fungal strain was immobilized on polyurethane foam cubes of 2 cm^3. After the pre-
139 cultivation on MEA, *B. adusta* was homogenized under sterile conditions in 9.0 g/L NaCl, and
140 inoculated into 1L flasks containing glucose and yeast extract liquid medium (GLY = 5.0 g/L glucose;
141 1.9 g/L yeast extract) and 2 cm^3 polyurethane foam (PUF) cubes. For each cube, 1.5 mL of homogenate
142 was added. Flasks were incubated in agitation for one week. After 7 days, the immobilization of the
143 fungus was complete and the cubes were used for batch and continuous experiments.

144 Old LFL was collected from Brady Road Municipal facility, Winnipeg, Canada and stored at + 4 °C.
145 Sampling was performed in the same well during all the experiments described in this study. LFL
146 chemical characterization was carried out before the beginning of each test (Table 1). The cellulose
147 used in this study is actually a waste material obtained from paper beverage cups (Tim Hortons,
148 Canada Beverage-Cup, BCC) after milling, and the cellulose content was estimated to be 86% (Agbor
149 *et al*., 2011).
150 2.2 Calibration curve of cellulose solubilisation
151 As a preliminary step for the set-up of cellulose containing bioreactor (Rc), a solubilisation curve of
152 cellulose was plotted for COD measurement using ordinary least squares linear regressions. COD was
153 measured according to Standard Methods for Examination of Water and Wastewater (SMEW, 20th
154 Edition), using dichromate method and HACH spectrophotometer DR2800. Soluble COD, sCOD was
155 measured after samples filtration, using Whatman filter papers grade 1. The cellulose concentrations
156 used to plot the curve were: 0.5, 1.0, 2.5 and 5.0 g/L. For each cellulose concentration, a stock solution
157 of 50 mL of BCC cellulose was prepared in deionized water and poured into Polypropylene Centrifuge
158 Tubes (50 mL volume). After standing overnight, the tubes were centrifuged for three cycles of 15
159 minutes at 14000 g. COD was measured in the stock solutions.
160 In order to ensure sample homogeneity, COD measurements were performed using the entire
161 cellulose stock volume, and the average COD value for each concentration was plotted in the
162 calibration curve. For each stock solution, at least 20 COD measurements were carried out.
163 2.3 Batch experiments: Cellulose at diverse concentrations
164 MnP production and COD removal were investigated using either a “complex sugar”, milled
165 cellulose, at diverse concentrations, or later on, a simple sugar, glucose and malt extract, as the co-
166 substrates. In the batch tests with milled cellulose as co-substrate, immobilized biomass was used. The
167 milled cellulose was prepared as described in Section 2.1 above. MnP activity was determined

spectrophotometrically (Biotek Powerwave Xs Microplate Spectrophotometer) by measuring at 590 nm dimethylaminobenzoic acid/3-methyl-2-benzothiazoline hydrazone hydrocloride (DMAB/MBTH), in 0.1 M succinate lactate buffer, pH 4.5, at 25 °C (Vyas *et al.*, 1994).

Each flask contained 7 PUF cubes, embedded with *B. adusta*, and 160 mL of LFL. Cellulose, previously sterilized by autoclaving it at 121 °C, 20 psi, was added in the 4 trials, using 0.5, 1.0, 2.5, and 5.0 g/L concentrations. The trials were incubated at 25 °C for 10 days with shaking (150 rpm). Unseeded controls, without fungal inoculum, containing 0.5, 1.0, 2.5, and 5.0 g/L of cellulose were performed and kept in the same experimental conditions. In all the flasks, pH was adjusted to 4.5, using H_2SO_4 (10%), before starting the experiment.

COD removal was measured and the estimation of cellulose solubilisation was carried out as the difference between COD values in the flasks during the treatment and COD value of 100% raw LFL, assuming that the increase in COD was mainly due to cellulose solubilisation. An estimation of solubilized cellulose in mg/L was based on the theoretical values of the calibration curve.

Color removal during the treatment was also evaluated spectrophotometrically. The decolorization percentage (DP) during the treatment was determined spectrophotometrically as the decrease of the spectrum area in the visible range (380-760 nm) with respect to the initial values (T0).

2.4 Batch tests: The effect of leachate organic load on MnP activity, COD, and sCOD removal

The effect of leachate organic load on MnP activity, COD and sCOD removal, using 2.5 g/L of cellulose, was evaluated. The test was performed using i) undiluted LFL and ii) 50% LFL. Fungal biomass was prepared by growing 10 plugs of about 1-2 mm in GLY liquid media for one week. At the end of the growth, the GLY was replaced with leachate (160 mL) and 2.5 g/L of cellulose were added. Cellulose sterilization, pH adjustment, controls preparation and incubation conditions followed the same protocol described previously in section 2.3. MnP was measured as described in Section 2.3. COD and sCOD were measured as described above Section 2.2.

192 2.5 Batch tests: Comparison with different co-substrates

193 MnP production by *B. adusta*, immobilized in PUF, was also evaluated using malt extract (1.0 g/L)
194 and glucose (1.0 g/L). The two co-substrates were added separately, and three independently replicated
195 experiments (i.e. three separate flasks) were performed for each condition. The same number of
196 unseeded controls, without fungal inoculum, was carried out. Fungal growth, immobilization and
197 incubation were performed as described above Section 2.1. MnP was measured as described in Section
198 2.3. COD and sCOD were measured as described above Section 2.2.

199 2.6 Experiments with continuously fed packed-bed bioreactors: Reactor design and operation

200 In this study, two packed bed bench-scale reactors were used (Figure 1) with a total volume of 5.0 L
201 and a working one of 4.5 L. The reactors were equipped with pH probes and controllers, which were
202 set at pH 6.0. The pH was maintained using 10% sulfuric acid. On the reactor's bottom, a diffuser,
203 connected with an air pump, provided air continuously with a flow of 2.0 L/min. A polyethylene cage
204 containing PUF cubes colonized by *B. adusta* MUT 2295 was fixed to a rotating shaft (about 5.0 rpm/
205 minute). Sixty (60) immobilized cubes were added in each reactor. The reactors were connected to two
206 tanks and two pumps (ISMATEC Reglo ICC, Digital Peristaltic Pump) for inlet and outlet. The
207 hydraulic retention time (HRT) was 72 hours (hrs), with a pump flow of 6.9 mL /min for influent and
208 effluent. A cycle of 6 hrs was employed. In every cycle reactors were fed with 416 mL of influent
209 during one hour; the same amount was discharged during the outlet that lasted also 1 hour. A lag time
210 of 4 hours occurred between the end of the discharge and the beginning of the following feeding.
211 Reactors were kept in a controlled temperature room (20 – 25 °C). Two different co-substrates were
212 used. Cellulose (0.5 g/L) was added in one reactor (Rc), inside the cage, as co-substrate at the start-up
213 of the reactor. LFL from Brady Road Landfill site was diluted at 33% using deionized water. Glucose,
214 0.5 g/L was added in the other reactor (Rg) by adding it directly inside the reactor, and LFL was diluted

215 50%. An additional co-substrate (glucose 0.5 g/L) was put directly in both the reactors after 75 days
216 and 53 days for Rg and Rc, respectively.
217 2.7 Experiments with continuously fed packed-bed bioreactors: Reactor monitoring
218 Grab samples were collected from the outlet tank daily and from the inlet tank in correspondence of
219 feed preparation that occurred weekly. COD and sCOD were estimated as parameters of treatment
220 efficiency. Ammonium nitrogen, nitrates and nitrites concentrations were measured in influent and
221 effluent. COD was measured according to Standard Methods for Examination of Water and
222 Wastewater (SMEW, 20th Edition), as described in Section 2.3. Removal Efficiency (RE) for COD and
223 sCOD was calculated as the percentage removed at a specific timing compared to the initial value at
224 T0. Particulate COD, pCOD, was calculated as the difference between COD and sCOD. The Biological
225 Oxygen Demand (BOD_5, 5 days) was measured according to SMEW. Ammonium nitrogen (NH_4^+-N),
226 nitrites (NO_2^--N) and nitrates (NO_3^--N) were measured via a flow injection analyzer (Quick Chem
227 8500, LACHAT Instruments). Samples for ammonium nitrogen, nitrites and nitrates were previously
228 filtered using Whatman filter papers grade 1.
229 **3.0 Results and discussion**
230 3.1 Batch experiments
231 Due to cellulose insolubility and consequent difficulties in measuring cellulose COD, a calibration
232 curve was plotted to calculate the concentration of cellulose solubilized during fungal treatment. COD
233 were measured in four stock solutions containing 0.5, 1.0, 2.5, and 5.0 g/L of cellulose, in 50 mL. COD
234 values increased linearly with cellulose concentration with $R^2 = 0.9859$, a slope of 1.059 and standard
235 deviations between 5 and 1246.
236 In *B. adusta* cultures with LFL and cellulose as co-substrate, MnP production was evaluated as a
237 function of cellulose concentration. The LFL used in this study was previously analysed (Table 1) and

238 showed the typical characteristics of old LFLs, with high refractory carbon load and high ammonium
239 nitrogen concentration.
240 The results of MnP activity and COD trends using different concentrations of cellulose are shown in
241 Figure 2A and Figure 2B, respectively. The enzymatic activity increased slightly during the first 48 hrs
242 of incubation in all trials, except in the trial with 1.0 g/L cellulose, in which MnP activity showed lower
243 enzymatic activity. However, greater MnP activity was detected in presence of 1.0 g/L at 96 hrs and
244 this was positively correlated with a decrease in COD (Figure 2B), which can be explained as partial
245 consumption of the BCC solubilized in LFL.
246 The greatest MnP activity was detected in the presence of 2.5 g/L of cellulose. MnP ranged from 9.6
247 U/L at 48 hr to approximately 26.0 U/L from 96 to 240 hrs (Figure 2A). The increase in the enzymatic
248 activity in 2.5 g/L trial was associated with a decrease in COD value, which can presumably be related
249 to a partial consumption of previously solubilised cellulose. Correlation of higher enzymatic activity
250 with co-substrate consumption was also reported by Saetang and Babel (2010). These authors
251 described, generally, that with fungi, co-substrate consumption is followed by the production of
252 secondary metabolites and extracellular enzymes for biodegradation.
253 The results observed in the trials with 1.0 and 2.5 g/L of cellulose suggest that the release of
254 cellulose due to solubilisation and its consequent consumption could enhance the production of MnP in
255 *B. adusta*. Since COD increases in the trials with 0.5 and 1.0 g/L of cellulose were higher than the
256 corresponding theoretical COD values of 0.5 and 1.0 g/L of cellulose (568 and 1052 mg/L), we can
257 expect that a total solubilisation of the added cellulose occurred in these flasks. In addition, in both
258 trials, COD reductions were higher than theoretical COD values of 0.5 and 1.0 g/L of cellulose,
259 suggesting that solubilized cellulose was consumed during the treatment.
260 In contrast, in the trials with 2.5 and 5.0 g/L of cellulose, the increase in COD, detected during the
261 treatment, represented around 57% and 68% of the corresponding theoretical COD values of 3217 and

5385 mg/L of cellulose, indicating that an incomplete solubilisation occurred and residual particulate cellulose was present in the trials.

Considering the COD reductions in 2.5 and 5.0 g/L, it is evident that not all the cellulose available was consumed, since the reductions during the whole period covered around 76% and 88%, of the COD increase values observed in the two trials (1831 and 3657 mg/L, respectively). From Figure 2B, it is possible to compare COD trend in the trials inoculated with *B. adusta* with the unseeded controls. The estimation of COD increases and consequent COD reductions resulted in higher values of both cellulose solubilisation and consumption in the inoculated trials, compared to the unseeded ones in all the concentrations tested. Although is not possible to discriminate LFL degradation from cellulose consumption, it is worth mentioning that the presence of *B. adusta* led to higher rate of cellulose solubilisation and COD decrease, in comparison to unseeded controls.

The enzymatic production was positively correlated with cellulose concentration at 2.5 g/L, while at 5.0 g/L cellulose, the activity was clearly lower and there was no correlation between enzyme activity and COD reduction. In addition, in this specific experiment, an exact quantification of cellulose solubilisation (allowing discrimination of eventual COD decreases due to degradative processes) could provide a better understanding of the ability of the fungus to exploit cellulose. The pattern observed is not surprising, considering *B. adusta* ability to degrade lignin and that other authors have previously reported its cellulolytic properties. For example, Quiroz-Castañeda *et al*. (2009) assessed the enzymatic production by *B. adusta* (UAMH 8258) using carboxymethylcellulose (CMC)ase in wheat straw agar medium and detected higher MnP activity levels compared to other fungi studied in literature. From this results, we can conclude that the fungal strain could exploit milled cellulose to induce the production of MnP and that the highest MnP activity was detected using 2.5 g/L of cellulose although, with this cellulose concentration, incomplete solubilisation and cellulose consumption occurred.

Color removal during the experiment is represented in Figure 2C. A slight decrease in the color was

observed in all treatments by 24 hrs. However, further significant decolourisation was only observed in the 2.5 and 5.0 g/L cellulose treatments. In particular, in the 2.5 g/L trial, the greatest color removal was detected after 96 h, which corresponded to a sharp increase in production of MnP. This result could suggest the presence of a degradative process carried out by the fungus, since MnP has been previously indicated as the major enzyme involved in LFL decolourisation (Tigini *et al*., 2013).

In the 5.0 g/L cellulose trial, it is not possible to exclude a partial biodegradation of LFL since enzymes were observed during the whole experiment, although the correlation of MnP peaks and decolourisation is less evident, since the enzymatic production was lower. Nevertheless, a close correspondence between enzymatic activity and decolourisation is not needed to ascribe biodegradation to peroxidases. Actually, these enzymes are very unstable and readily inactivated by their substrate, but they activate a chain reaction, which propagates the oxidation over peroxidase inactivation (Anastasi *et al.*, 2010). This can explain the greater level of color removal in the 5.0 g/L of cellulose trial. Even if adsorption of pigments to fungal biomass could be an alternative explanation of the observed pattern, the biomass did not show a markedly coloured aspect after the trial, thus this phenomenon had certain a marginal role.

The effect of LFL dilution was assayed using 2.5 g/L of cellulose as co-substrate. This experiment was performed using free suspended biomass, allowing also the comparison of MnP activity in 100% LFL with 2.5 g/L of cellulose in suspended versus immobilized cultures. The results of 2.5 g/L of BCC in 100% and 50% LFL are shown in Figures 3A and 3B. Parameters of LFL used in this experiment were as following: pH 8.4, COD 1630 (mg/L), sCOD 1620 (mg/L), ammonium nitrogen 940 (mg/L), BOD_5 150 mg/L and 0.090 BOD_5/COD. After dilution, 50% LFL COD and sCOD were 814 ± 23 (mg/L) and 787 ± 24 (mg/L), respectively.

Figure 3A reveals that the MnP activity started very early in both trials, reaching 8 U/L after 4 hrs in

309 50% LFL and 4 U/L in 100% LFL, although no significant differences were detected between the
310 two data sets. Extended analysis of MnP activities from 24 to 240 hrs (Figure 3B) showed that MnP
311 activities were similar in both treatments, with no statistically significant differences (test-t, $p > 0.05$).
312 Since a slightly higher MnP production trend was found in 100% LFL, it is reasonable to hypothesize
313 that the enzymatic activity of the fungus could be a stress response due to the characteristics of LFL,
314 such as high organic load and/or high ammonium nitrogen concentration. Indeed, several authors have
315 reported an increased MnP activity in high N conditions (Kaal *et.al.*, 1995, Şeker *et al.*, 2008; Anastasi
316 *et al*., 2010).
317 It has been observed that LFL characteristics play a crucial role in determining interference on the
318 enzymatic productions and variability among species and strains can be observed (Ellouze *et al*., 2009;
319 Kalčíková *et al*., 2014). Indeed, LFL used in this study contained approximately 1.0 g/L of ammonium
320 nitrogen, which is normally problematic to microorganisms. Our understanding from the detected
321 pattern is that the reduction of organic load and ammonium nitrogen of LFL did not enhance MnP
322 production in *B. adusta*. This result is closer to the pattern found by Ellouze *et al*. (2009) since these
323 authors reported MnP reductions starting from 2 g/L of ammonium nitrogen and only a limited delay
324 was found with 1.0 g/L ammonium.
325 The trends in reduction of COD trend in 100% and 50% LFL are showed in Figure 4. The
326 differences between COD levels in 100% LFL in the absence of cellulose versus the presence of
327 cellulose, that cellulose was partially solubilized at the beginning of the experiment. Indeed, only 395
328 mg/L of COD due to cellulose was present, which correspond to a theoretical concentration of
329 solubilized cellulose of 260 mg/L. The decrease in COD values between 0 and 48 h is presumably due
330 to cellulose consumption, which is positively associated with a peak in MnP activity, indicating a
331 benefit of the fungus due to the co-substrate utilized. Between 48 and 144 hrs, a second step of

332 solubilization occurred, which was followed by a second co-substrate consumption at 192 hrs. The
333 detected decrease in COD was associated with an increase in the enzymatic production up to 24 U/L.
334 The diluted LFL has a similar pattern, although no co-substrate consumption was detected in the
335 first 48 hrs of treatment and, therefore, a positive correlation between MnP peak and cellulose
336 consumption was not observed. The solubilization of cellulose increased, reaching its maximum value
337 at 144 hrs with 1596 mg/L of COD, attributable to cellulose, and corresponding to a theoretical
338 concentration of 1.4 g/L of cellulose (56% of the 2.5 g/L cellulose introduced). The COD at 192 hrs
339 was decreased of 616 mg/L. Although the reduction could not be totally explained by co-substrate
340 consumption because of the lack of information that can exclude any biodegradative process of
341 leachate occurred during the treatment, we hypothesize a partial co-substrate consumption, which is
342 positively correlated with the increase in MnP level from 10 to 21 U/L.
343 In the last part of the experiment, COD decreased and MnP production drastically dropped from 21
344 to 9 U/L. The data collected do not allow to discriminate the COD decrease due to biodegradation and
345 the one of co-substrate consumption; however, the final value of COD at the end of the treatment
346 indicate an incomplete solubilization. In addition, assuming that the COD reduction was totally
347 attributable to cellulose consumption and no degradative process occurred, the theoretical cellulose
348 consumption was lower than the COD concentration solubilized from the beginning of the experiment
349 corresponding to residual 584 mg/L of solubilized cellulose unutilized, which represents 435 mg/L of
350 cellulose. MnP activity in 100% LFL using suspended biomass was comparable with the one detected
351 with *B. adusta* immobilized in PUF cubes, since in both cases the maximum MnP activity was 27 U/L.
352 However, it is possible to observe that in the suspended culture trials, the peak occurred after only 48
353 hrs of treatment, earlier compared to 192 hrs required to achieve the same activity in immobilized
354 cultures. In contrast, other studies reported higher enzymatic activities in immobilized cultures
355 compared to suspended cultures (Rodriguez-Couto *et al*., 2009; Spina *et al*., 2012). Due to the

difficulties in cellulose solubilization, we can hypothesize that the use of free suspended biomass could facilitate the contact of the fungus to the co-substrate, enhancing enzymatic production.

Malt extract and glucose were added to LFL to evaluate their possible use as co-substrates for LFL treatment with the selected fungal strain. In Figure 5, a summary of MnP activities in the different conditions investigated is represented. MnP activities were detected using both co-substrates, malt and glucose, although slightly higher activities were observed using malt extract alone, which is commonly used for culturing fungi. From all these batch tests, it is possible to conclude that the selected fungal strain, *B. adusta* MUT 2295, was able to exploit all the co-substrates, tested in this study, to produce MnP. However, when comparing MnP activity in all the conditions, it is possible to observe that earlier activity was detected in suspended cell cultures with cellulose compared with immobilized cells with cellulose, malt extract or glucose as co-substrate, providing further evidence that the use of suspended biomass, in the tested conditions, could enhance *B. adusta* ability of expressing MnP.

3.2 Experiments with packed-bed bioreactors

Experiments in continuous bioreactors were performed to estimate the efficiency of fungal treatment with *B. adusta* on longer term and under non-sterile conditions using cellulose and glucose as co-substrates in the reactor with glucose (Rg) and in the reactor containing cellulose (Rc), respectively. Initial batch tests (3.1) revealed total consumption of 0.5 g/L of cellulose during the treatment, and suggested that the use of this concentration in the initial phase of the bioreactor experiments could facilitate the quantification of organic removals due to biodegradative processes. The same co-substrate concentration (0.5 g/L) was added in the bioreactor with glucose (Rg). Parameters of LFL before dilution are reported in Table 2. LFL was diluted with deionized water before every feeding of the reactors in order to reduce the organic load, to optimize process efficiency.

After dilution, the COD and sCOD of 50% LFL in the Rg bioreactor, were 782 ± 20 mg/L and 726 ± 20 mg/L, respectively. Considering glucose addition as start-up, the initial COD and sCOD of the Rg

bioreactor were 1282 ± 72 mg/L and 1176 ± 201 mg/L, respectively. The initial COD and sCOD of 33% LFL in the Rc bioreactor, were 547 ± 17 mg/L and 535 ± 19 mg/L, respectively.

COD and sCOD removals achieved with the Rg bioreactor (glucose as co-substrate) are shown in Figure 6A. The bioreactor was operated for 99 days (approximately 33 times the HRT). Based on the observed total COD and soluble sCOD Removal Efficiencies (REs), the Rg bioreactor data may be subdivided in three phases. In the first phase, which covered the first three weeks, average REs of 51% and 48% were found for COD and sCOD, respectively. The maximum sCOD removal (53% = 695 mg/L of sCOD reduction) was observed after 20 days, and at the same time, the maximum COD removal was also observed, achieving 63% of COD reduction.

The second phase started at day 25 when a sharp decrease in RE was observed. The second phase did not show a linear pattern of RE and was characterized by fluctuations. The average RE was lower in the second phase than in the first phase, and corresponded to a decrease of 14% for both, COD and sCOD. The maximum sCOD removal in the second phase was 18% at day 50, corresponding to 133 mg/L of sCOD depletion, and at the same time, the maximum COD removal was also observed, reaching 22% of COD reduction.

The third phase started at day 60, with an increase in RE for both COD and sCOD. In the third phase, the average RE was 27% and 24% for COD and sCOD, respectively. An additional increase in RE was observed after glucose addition (0.5 g/L) at day 75. Increased RE lasted up to day 85, indicating that co-substrate dosage, although not continuous, could enhance process performance. The maximum removals, in the third phase, were observed after 80 days, achieving 51% and 44% for sCOD and COD reduction, respectively.

The concentration of Particulate COD, pCOD, in the effluent did not exceed 100 mg/L and did not present a clear increasing pattern compared to the influent. Since pCOD in the effluent can be due to, high rate of fungal growth or biomass loss from the cubes related to excessive stress levels, the pattern

of pCOD observed in the effluent suggests that fungal biomass was almost stable during the treatment. The highest pCOD value was 98 mg/L after 14 days of treatment and it can presumably be associated with an increase in microorganism's growth consequent to glucose addition.

Although, a stable RE pattern could not be detected in Rg, the increasing trend of the last part of the experiment suggests a progressive acclimation of the fungus and associated microorganisms to LFL. This results is associated with a negligible release of pCOD in the effluent, indicating that fungal biomass was stable during the treatment.

The trend of COD and sCOD removal of the reactor with cellulose are shown in Figure 6B. The reactor was operated for 69 days. As with the Rg bioreactor, the Rc bioreactor data may be subdivided in three phases. The first phase occurred within the first 20 days, and the average RE was 10%. The second phase occurred between day 20 and day 50. The sCOD RE increased between day 20 and 25, decreased slowly between day 25 and day 46, and then began to increase again up to day 50. The average RE in the second phase was 20%. In the third phase, the sCOD removal increase sharply after the addition of 0.5 g/L of glucose on day 53. The average RE in the third phase was 29%. Maximum COD and sCOD removal during the treatment were 54% and 51%, achieved after co-substrate addition at day 53 of treatment.

Important COD increases in the effluent, compared to the influent, were not detected, suggesting that the co-substrate was consumed during the treatment, as was previously observed during batch tests. Although several fluctuations, also in the reactor with cellulose, effluent pCOD was negligible compared to the amount of the inoculated fungal biomass. Moreover, it was similar to the amount recorded in the influent, suggesting that the biomass was stable during the treatment. Also in this case, a peak in pCOD was found after co-substrate addition at day 55 with 137 mg/L of pCOD, providing further evidence of biomass growth after co-substrate addition.

Although similar values of maximum sCOD removal in the two reactors were reached, it is

428 important to notice that they occurred in two different timing, suggesting that the complementary effect
429 of the two co-substrates could be exploited by adding glucose as start-up and cellulose in a second step
430 of the treatment. The results achieved in both reactors indicate that also non-continuous co-substrate
431 addition could led to satisfactory RE rates, reducing the costs of continuous co-substrate dosage.
432 Hence, sporadic co-substrate addition could be exploited to enhance RE, when necessary.
433 A complete understanding of the process occurred could be achieved through additional detailed
434 chemical characterization of LFL to assess eventual compounds rearrangements due to the degradative
435 process. Moreover, molecular characterization of the microbial components during the treatment could
436 allow to understand to which extent the fungus contributed to autochthonous microbial community.
437 To our knowledge, this study is the first report of fungal treatment toward old LFL performed in
438 continuous and under non-sterile conditions. Indeed, previous studies concerning fungal treatment
439 toward old LFL were performed on batch (Kalčíková *et al*., 2014).
440 The major drawbacks that limit scaling-up to larger volumes and full-scale fungal applications are:
441 i) a predilection the fungal biomass to generate low pH (Anastasi *et al*., 2010), ii) the requirement for
442 an additional carbon source; iii) the need for further steps for ammonia removal; and iv) the difficulties
443 in maintaining long-term efficiency in non-sterile conditions (Kalčíková *et al*, 2014). In addition,
444 fungal needs and responses to medium feeding, and the development of agitation/aeration rates and
445 methodologies that minimize the inhibition of hyphal growth and strengthen fungal metabolism are still
446 The first study in continuous of fungal treatment on LFL was reported on young LFL. Saetang and
447 Babel (2009) carried out continuous fungal treatment on young LFL according to different conditions,
448 reporting a maximum COD removal of 42% with 3.0 g/L of glucose and an increasing pattern in RE
449 during 20 days of experiment. This pattern is similar to the first leg of our experiment, using glucose,
450 although co-substrate nature and concentration differed within the two studies. The same authors
451 reported also 23% of COD removal without adding co-substrate in 4 cycles of treatment. This result

452 was similar to the second stage of both our experiments, in which co-substrate was probably depleted.
453 Experiments about aerobic granular sludge treatment of LFL collected from the same landfill in the
454 same timing of our study (Ren and Yuan, 2016) resulted in a COD removal of 50% using LFL
455 concentrations lower than 60% and 25% of COD removal with 70% of LFL. Considering that
456 microorganism's acclimation can result in higher performance, our REs have been achieved in
457 relatively short amounts of time. In addition, due to the lack of chemical characterization of the
458 compounds depleted in both treatments, a possible combined approach of aerobic granular sludge and
459 fungi can be evaluated to enhance RE of LFL. The results achieved in this study are promising
460 considering the nature of old LFL, characterized by low availability of organic load. A further step
461 would understanding which compounds have been degraded and if the treatment proposed in this study
462 could enhance the performance of conventional biological processes, including activated sludge and
463 aerobic granular one, on old LFL itself.

464 **4.0 Conclusions**

465 Enzymatic production and COD removal of *B. adusta* from old LFL were investigated as a function
466 of co-substrate and dilution, revealing the basidiomycetes capability to exploit cellulose, malt extract
467 and glucose as co-substrates. Continuous tests, with irregular co-substrate additions, showed a
468 significant increase in RE after the dosages, resulting in a maximum sCOD removal of 52% and 51%
469 after 20 and 54 days using glucose and cellulose as co-substrate, respectively. The treatment seems to
470 be promising and its possible exploitation to complement state of art technologies could be evaluated to
471 optimize recalcitrant compounds removal from old LFL.

472 **5.0 Acknowledgment**

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475 for providing the fungal strain.

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Figure Captions

Figure 1. Representation of the Packed-bed bioreactor design: 1) feeding tank; 2) pH controller; 3) pH probe; 4) acid solution to adjust pH; 5) mixer engine; 6) cage equipped with polyurethane foam carriers (PUF); 7) air-sparger; 8) aerator; 9) outlet tank.

Figure 2. Batch tests results using BCC 0.5, 1.0, 2.5, and 5.0 g/L. A) MnP activity during the test. Bars represented standard deviation (S.D.) among replicates; B) COD trend in the four trials, indicated as T, in the respective unseeded controls, indicated as C, and 100% LFL. BCC concentration is shown in g/L to discriminate the trials; C) Decolouration percentage (D.P.) during the test. Values are given as average among three replicates and bars are standard deviations (S.D.) of the means. Negative values should be considered as an increase in the spectrum area.

Figure 3. A) Enzymatic production (MnP) using suspended biomass and 2.5 g/L cellulose (BCC) during the first 6 hours of treatment; B) Enzymatic production (MnP) in the same conditions of (A) from 24 h until the end of the treatment. In a) and b) bars represented standard deviations (S.D.) of the means. .

Figure 4. COD trends (mg/L) in experiments with suspended biomass and 50% or 100% LFL, using 2.5 g/L of cellulose (BCC).

Figure 5. MnP enzyme activities tested in batch reactions with cellulose (BCC) versus glucose. *B. adusta* was immobilized in all the trials except BCC 2.5 g/L, LFL 50%, and LFL 100 %, where *B. adusta* was grown asa suspended culture. Bars represented standard deviations (S.D.) of the means..

Figure 6. A) COD and sCOD removal (%) during continuous treatment in the bioreactor with glucose. 1 and 2 indicate glucose (0.5 g/L) additions. Vertical lines show the different phases during reactor's operating time; B) COD and sCOD removal (%) during continuous treatment in the reactor with cellulose start-up. 1 indicates glucose (0.5 g/L) addition. Vertical lines show the different phases during reactor's operating time.

Table 1. Parameters of LFL used during batch experiments with cellulose. Values are given as average three replicates. Standard deviations (S.D.) are indicated in the third column, with minimum and maximum values in the fourth and fifth columns, respectively.

Parameter	Average	S.D.	Min.	Max.
pH	8.55	0.07	8.50	8.60
COD (mg/L)	2265	2.82	2263	2267
sCOD (mg/L)	2052	262.13	2238	1867
BOD_5 (mg/L)	175	35	150	200
BOD_5/COD	0.077	-	-	-
NH_4-N (mg/L)	930	198	790	1070

662 **Table 2.** Characterization of LFL used during the experiment with bioreactors. Values are given as
663 averages of three replicated experiments over the whole operating time. Standard deviations (S.D.) are
664 indicated in the third column, with minimum and maximum values in the fourth and fifth columns,
665 respectively

666

Parameter	Average	S.D	Min.	Max.
pH	8.5	0.14	8.4	8.6
COD	1585	108	1388	1761
sCOD	1471	99	1335	1676
BOD_5	175	35	200	350
NH_4-N	725	202	398	1070

667
668

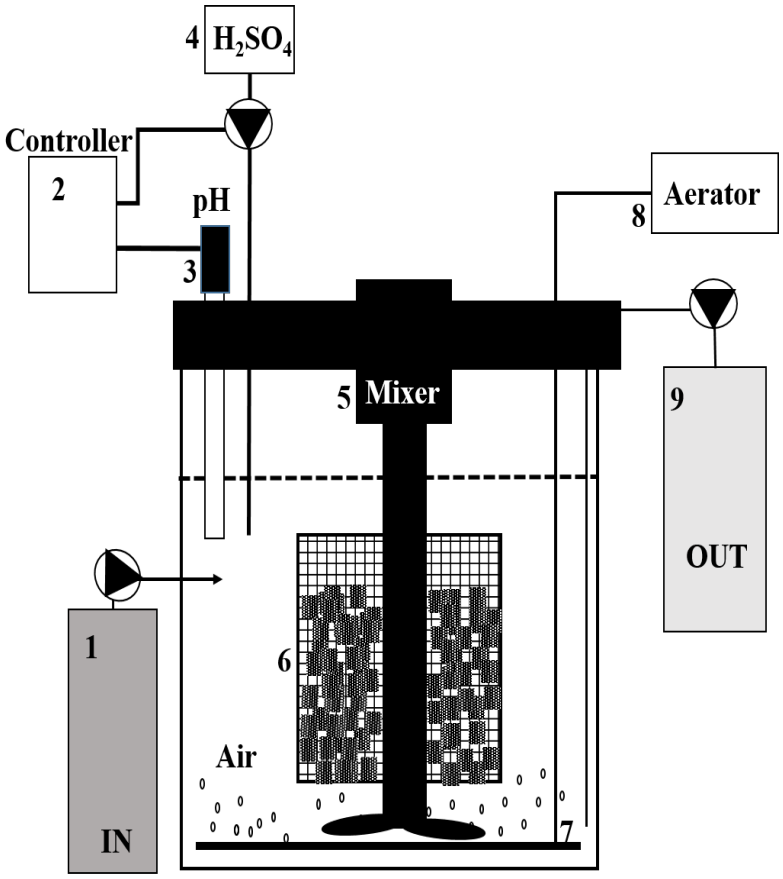
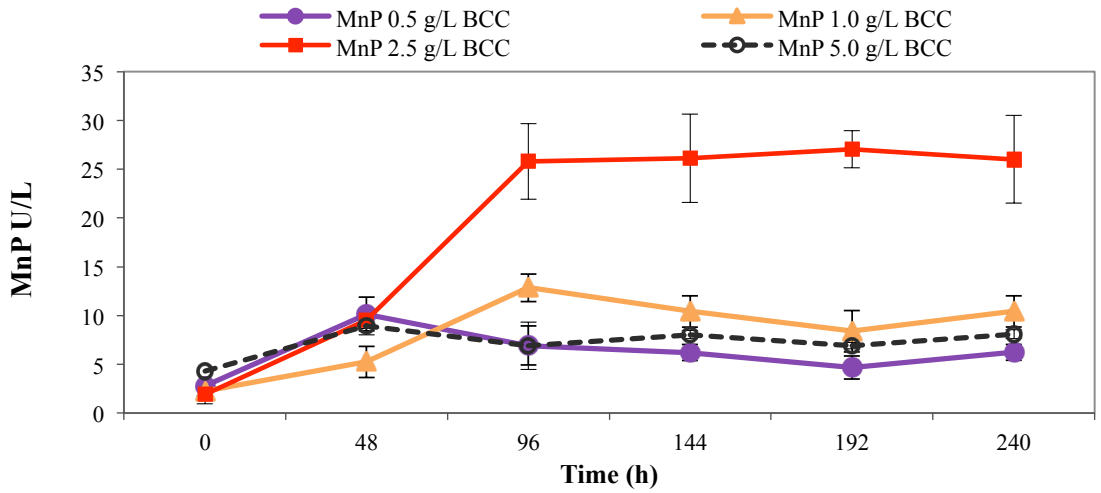
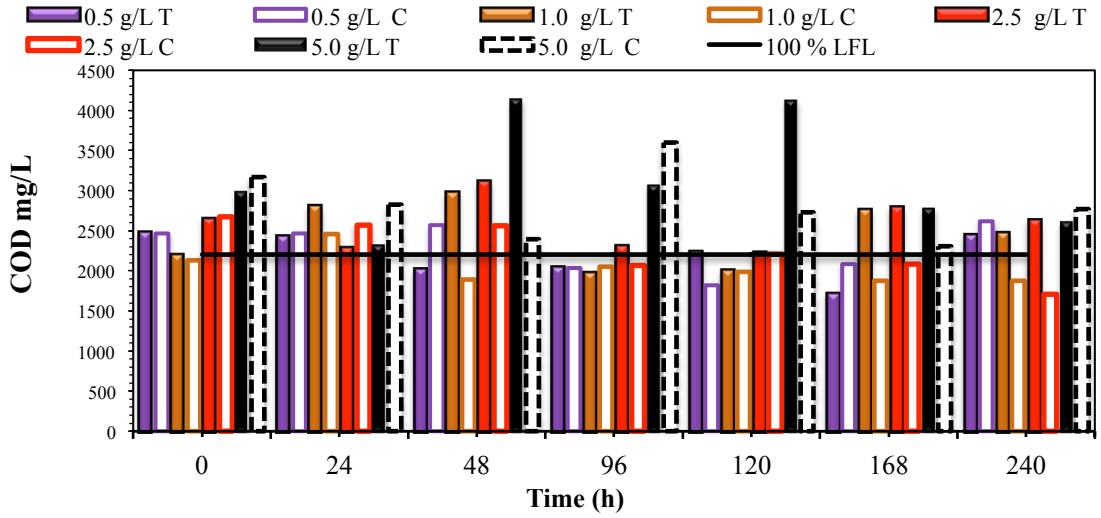


Figure 1.

A


MnP 0.5 g/L BCC
MnP 1.0 g/L BCC
MnP 2.5 g/L BCC
MnP 5.0 g/L BCC
MnP U/L
35
30
25
20
15
10
5
0
0
48
96
144
192
240
Time (h)

B


0.5 g/L T
0.5 g/L C
1.0 g/L T
1.0 g/L C
2.5 g/L T
2.5 g/L C
5.0 g/L T
5.0 g/L C
100 % LFL
COD mg/L
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
24
48
96
120
168
240
Time (h)

C

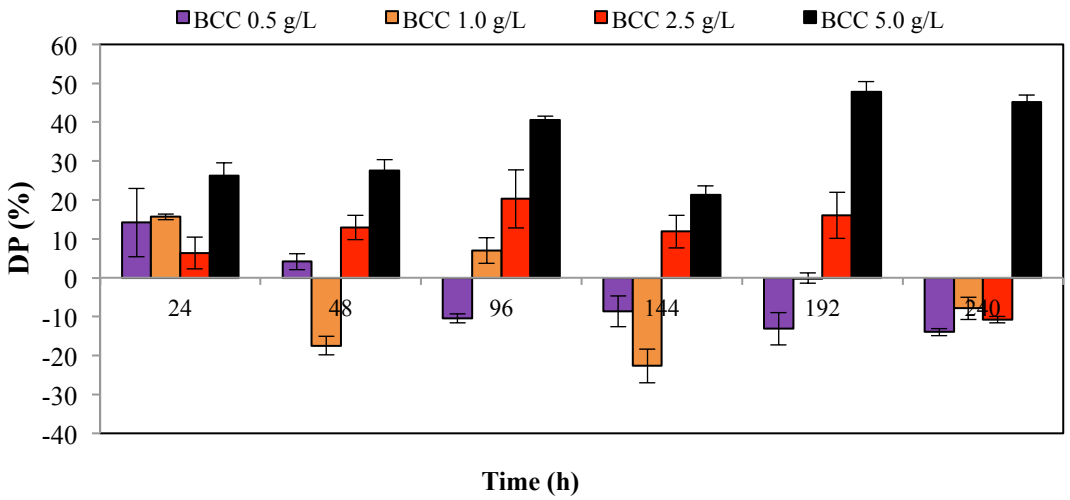
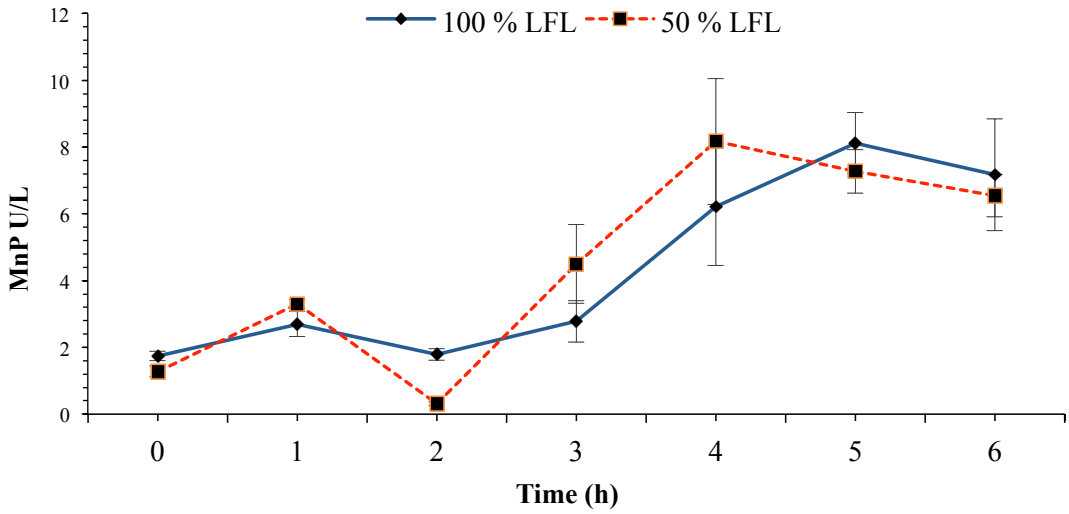

BCC 0.5 g/L
BCC 1.0 g/L
BCC 2.5 g/L
BCC 5.0 g/L
DP (%)
60
50
40
30
20
10
0
-10
-20
-30
-40
24
48
96
144
192
240
Time (h)

Figure 2.

A


100 % LFL
50 % LFL
MnP U/L
Time (h)
0
2
4
6
8
10
12
0
1
2
3
4
5
6

B

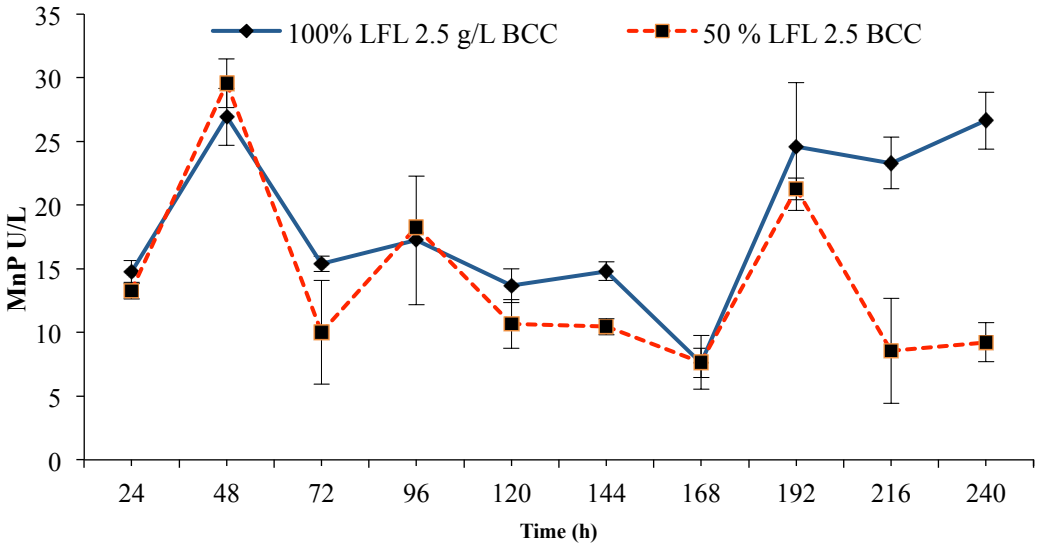
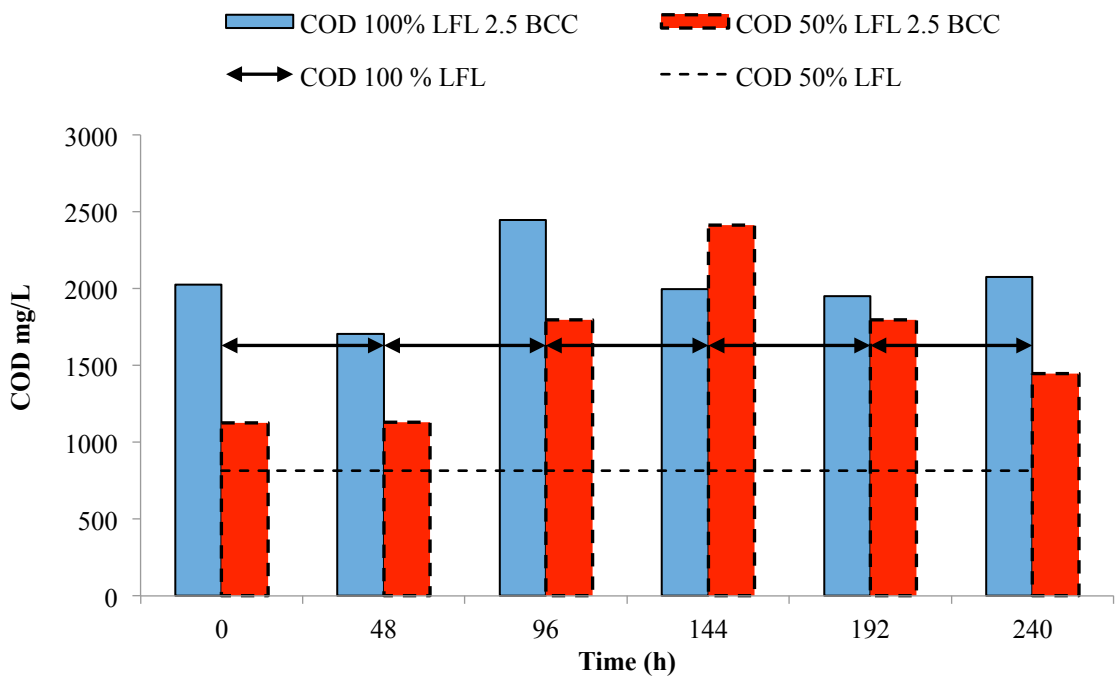

100% LFL 2.5 g/L BCC
50 % LFL 2.5 BCC
MnP U/L
Time (h)
0
5
10
15
20
25
30
35
24
48
72
96
120
144
168
192
216
240

Figure 3.



740

741 **Figure 4.**

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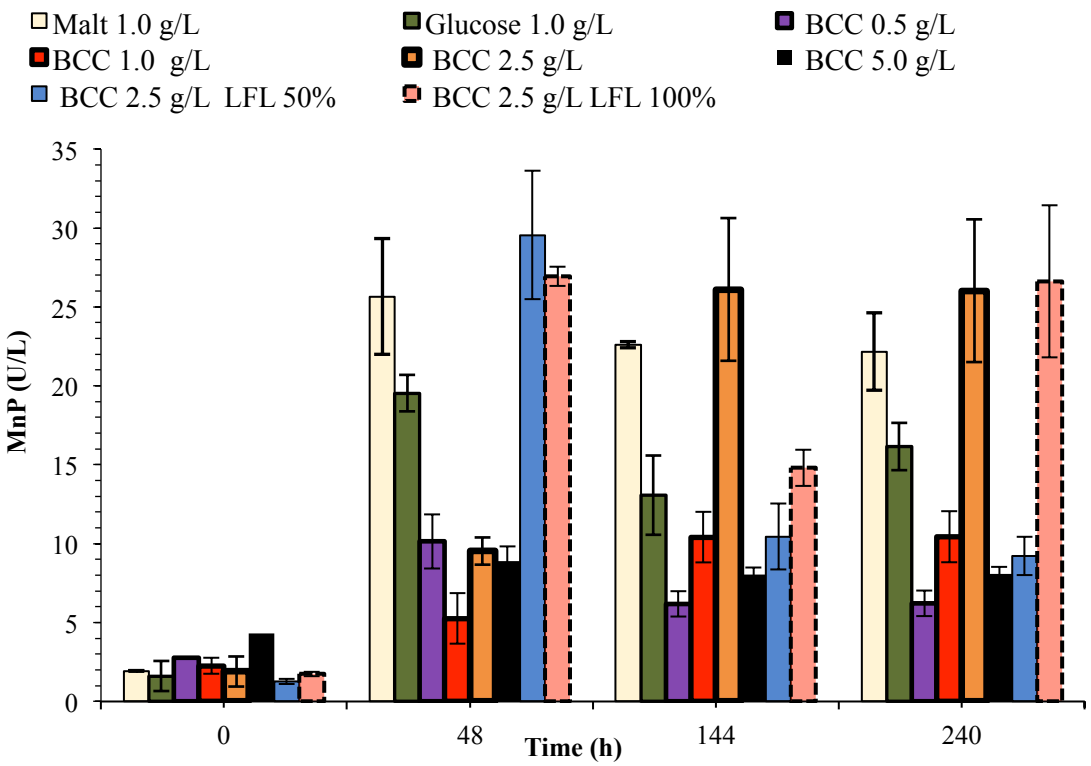

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746

747 **Figure 5.**

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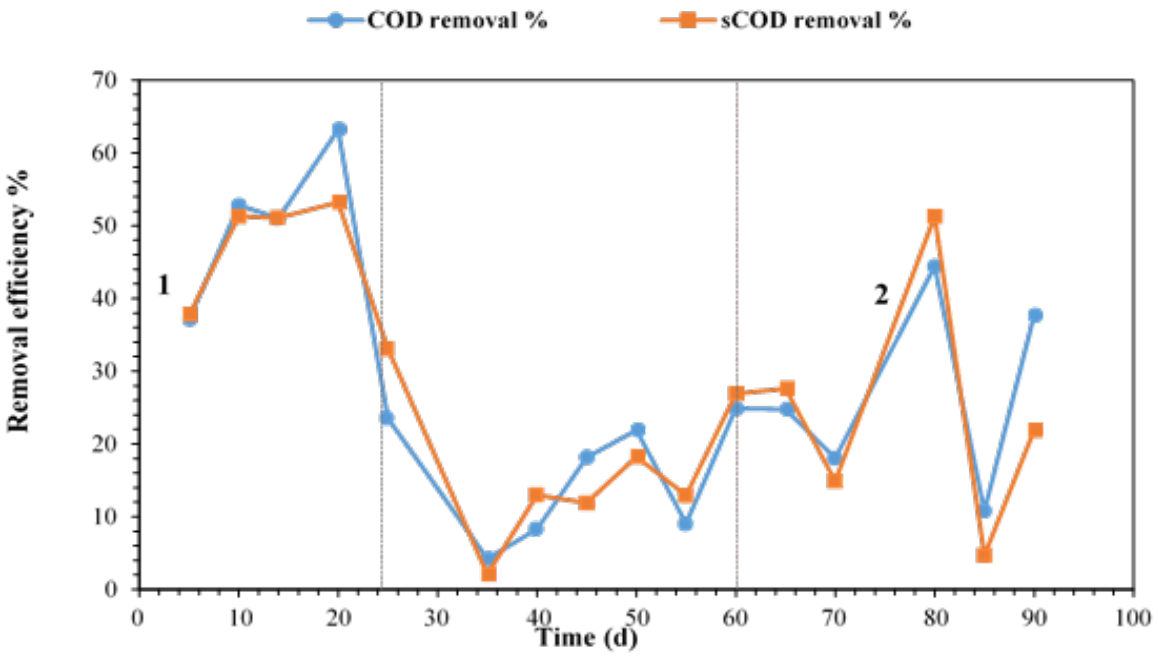
750 **A**



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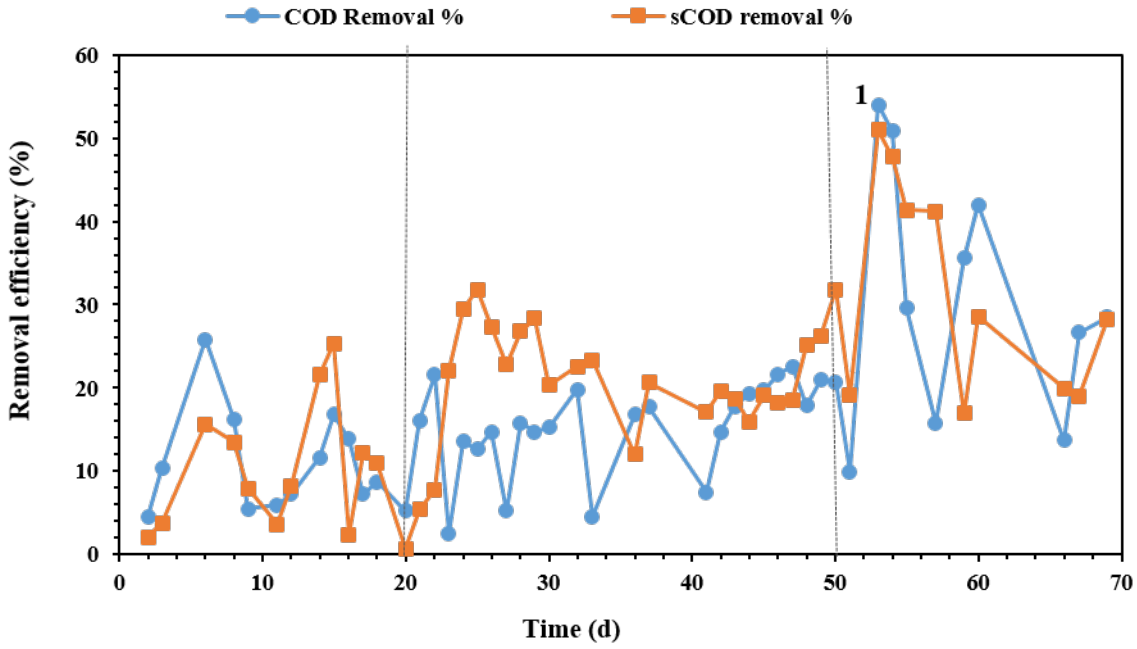
753 **B**



754

755 **Figure 6.**

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